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REVIEW

Insight into the Progress in CAR-T Cell Therapy and Combination with Other Therapies for Glioblastoma

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Abstract: Glioblastoma (GBM) is the most common malignant primary brain cancer in adults. It is always resistant to existing treatments, including surgical resection, postoperative radiotherapy, and chemotherapy, which leads to a dismal prognosis and a high relapse rate. Therefore, novel curative therapies are urgently needed for GBM. Chimeric antigen receptor T (CAR-T) cell therapy has significantly improved life expectancy for hematological malignancies patients, and thus it increases the interest in applying CAR-T cell therapy for solid tumors. In the recently published research, it is indicated that there are numerous obstacles to achieve clinical benefits for solid tumors, especially for GBM, because of GBM anatomical characteristics (the blood–brain barrier and suppressive tumor microenvironment) and the tumor heterogeneity. CAR-T cells are difficult to penetrate blood–brain barrier, and immunosuppressive tumor microenvironment (TME), which induces CAR-T cell exhaustion, impairs CAR-T cell therapy response. Moreover, under the pressure of CAR-T cell therapy, the tumor heterogeneity and tumor plasticity drive tumor evolution and therapy resistance, such as antigen escape. Nonetheless, scientists strive for strategies to overcome these hurdles, including novel CAR-T cell designs and regional delivery. For instance, the structure of multi-antigen-targeted CAR-T cells can enrich CAR-T accumulation in tumor TME and eliminate abundant tumor cells to avoid tumor antigen heterogeneity. Additionally, paired with an immune modifier and one or more stimulating domains, different generation of innovations in the structure and manufacturing of CAR-T cells have improved efficacy and persistence. While single CAR-T cell therapy receives limited clinical survival benefit. Compared with single CAR-T cell therapy, the combination therapies have supplemented the treatment paradigm. Combinatorial treatment methods consolidate the CAR-T cells efficacy by regulating the tumor microenvironment, optimizing the CAR structure, targeting the CAR-T cells to the tumor cells, reversing the tumor-immune escape mechanisms, and represent a promising avenue against GBM, based on multiple impressive research. Moreover, exciting results are also reported to be realized through combining effective therapies with CAR-T cells in preclinical and clinical trials samples, have aroused inspiration to explore the antitumor function of combination therapies. In summary, this study aims to summarize the limitation of CAR-T cell therapies and introduces novel strategies to enhance CAR-T cell function as well as prospect the potential of the therapeutic combination.

Keywords: CAR-T, GBM, novel strategies, therapeutic combination

Introduction

Glioblastoma (GBM) is a highly aggressive and incurable brain tumor and is one of the most lethal human cancers.[1,2] Compared with other solid tumor, therapeutic improvements for GBM have been minimal over the past 2 decades.[3] Tumor-treating fields (TTFields) is the only treatment that has been shown to improve clinical outcome.[4] Current Food and Drug Administration (FDA)-approved treatments for GBM are limited, which are majorly comprised of surgical resection, radiotherapy, chemotherapy, and TTFields, with a median survival following diagnosis of approximately 20.5 months.[4,5]

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In recent years, immunotherapy strategies have revolutionized the treatment of multiple tumors, increasing the hope for GBM therapy.[6] Unfortunately, GBM poorly responses to current immunotherapies. The extrinsic components that are native to the brain, such as the blood–brain barrier (BBB) and suppressive tumor microenvironment (TME), and intrinsic mechanisms, such as molecular heterogeneity which aid in immune evasion, are the main contributors to GBM resistance to various immunotherapies. Functional and tumor-specific cytotoxic T lymphocytes drive the adaptive immune response to the malignant tumor. Thus, the induction of their antitumor activities is the ultimate goal of all immunotherapies.[7,8] Compared with the tumors, sensitive to immunotherapy, such as non-small-cell lung cancer and renal cell carcinoma, there are scarcity of T lymphocytes, whereas abundant tumor-associated macrophage (TAMs) in GBM microenvironment, because of the low immunogenicity and physical barriers. That is the reason that the efficacy of immune checkpoint inhibitors (ICIs) is limited in GBM. There are rare T cells that can be activated by ICIs in GBM TME. Furthermore, the immunosuppression TME impairs a beneficial response. Moreover, GBM is with a highly immunosuppressive TME, and the immunosuppressive myeloid cells/microglia account for the most abundant cellular constituents, while $CD8^+$ T cells are the most cell subtype in immunotherapy-sensitive lung adenocarcinoma.[9,10] The immunosuppressive cytokines, immune factors and myeloid cells/microglia can alter infiltrative T cells into exhaustion forms.[11,12] Additionally, targeted and immunotherapy for GBM largely fail due to the complexities arising from intratumoral molecular heterogeneity.[13] Glioma stem cells (GSCs), which are characterized by self-renewal, multilineage differentiation potential, play critical role in resistance to treatment and tumor heterogeneity.[14,15] GSCs will evolute to other forms to adapt to the various treatment stress, and drive tumor treatment resistance. These features are the GBM specific factors that promote the failure of novel GBM treatments.

For decades, scientists invent chimeric antigen receptors (CARs) for expression on immune cells, among which T cells are the most common.[16] The advantage of CAR-T cells is to reprogram abundant T cells, which aim to overcome the spare infiltration in GBM TME, against specific antigens, initiating a new era of personalized cancer therapy.[17] While CAR-T cells have also been limited by a lack of sufficient delivery to the brain.[18] For example, Maus et al report that intravenous delivery of CAR-T cells does not significantly prolong survival in GBM mice models, and intraventricular infusion with CAR-T cells is efficacious against GBM.[19] Currently, regional delivery of CAR-T cells, radiotherapy and utilization of low-intensity pulsed ultrasound to open BBB are novel strategies to enhance CAR-T in situ delivery. Besides, the design of multi-targets or targeting GSCs CAR-T cells and combination of other therapies could eliminate adequate tumors cells and regulate immunosuppressive TME to avoid tumor recurrence and CAR-T inactivate. In the structure of CAR-T, there are four major components, including an extracellular target antigen-binding domain, a hinge region, a transmembrane domain, and one or more intracellular signaling domains.[17] Currently, based on the various CAR-T cell structures, CAR-T cell has developed to the fifth generation, such as introduce of pro-inflammatory cytokines and signaling binding motif to sustain both long-term CAR-T cell proliferation and reverse suppressive TME.[20,21]

This review aims to describe the immune and molecular characteristics of GBM, which cause resistance to CAR-T cell therapy. Additionally, to overcome the current limitation, the development of genetically modified CAR-T cells and the combination of CAR-T cell therapy with other antitumor therapies are summarized.

The Characteristics of TME and Molecular Features in GBM

The suppressive TME around tumor cells is an insurmountable barrier attenuating the efficacy of CAR-T cell therapies in solid tumors. Besides, compared to other solid tumors, GBM patients rarely benefit from current immunotherapies, due to the special suppressive TME, and there is no FDA-approved immunotherapy in GBM.[22] First of all, as a highly selective physiologic barrier, the BBB can dampen immune cells' infiltration into GBM, thus resulting in a relative "cold tumor".[23] Similarly, the unique anatomical boundary is also seen to be a huge break for CAR-T cell delivery. Secondly, complex GBM TME, including abundant endothelial cells, fibroblasts, macrophage, and suppressive cytokines, together with sparse T lymphocytes,[24,25] is quite distinct from other cancer types. The hostile GBM TME can educate infiltrative CAR-T cells into exhaustive ones. Thirdly, given the theory of "seeds" (cancer cells) and "soil" (the organ that cancer cells grow), it is reported that the various tumor histology (seeds) rather than intracranial location (soil) can induce T cells exhaustion and that T cells exhaustion is significantly severe among malignant glioma, in comparison with melanoma, breast, and lung.[26] This interesting finding highlights that GBM can strongly impair T cells' function. In summary, key challenges restricting efficacy and/or preventing the broad application of CAR-T cell therapies for GBM, are the restricted trafficking to, infiltration into, and activation of GBM TME.[27] Through using advanced CAR-T cell technologies, these limitations may be better solved. Integration of molecular studies has led to more refined diagnoses and allows further clarification of the pathophysiologic processes that contribute to malignant phenotype and immunosuppressive TME.[28] The most recent update of the World Health Organization (WHO) classification of brain tumors in 2021 introduces single nucleotide substitute (isocitrate dehydrogenase 1/2 and telomerase reverse transcriptase mutation), chromosomal abnormalities (cyclin-dependent kinase inhibitor 2A/B homozygous deletion, 1p/19q deletions, chromosome 7 gain/10 loss, and epidermal growth factor receptor amplifications), and promoter methylations (O6 methylguanine DNA methyltransferase) into the diagnosis of glioma.[29,30] In addition, according to bulk expression profiles, research reveals that GBM samples could be classified into one of three states-proneural, classical and mesenchymal, with distinct genetic events, such as platelet derived growth factor receptor alpha alterations in proneural, epidermal growth factor receptor (EGFR) amplification in classical, and neurofibromatosis type 1 (NF1) mutation in mesenchymal.[31–33] These cellular state is plasticity, under treatment stress. These novel markers are reported to induce the immunosuppressive TME. For instant, the activated EGFR pathway (about 50% in GBM) induces the PD-L1 expression to enhance the immune escape,[34] and T cells are depleted in EGFR-amplified GBM samples.[35] Additionally, alpha-thalassemia/mental retardation syndrome X-linked (ATRX) inactivation contributes to oncogenesis and knock-down ATRX could induce PD-L1 and an immunosuppressive cytokine/chemokine profile, such as interleukin 6 (IL6), interleukin 8 (IL8), and interleukin 33 (IL33), in vitro and vivo.[36] Moreover, T cells enrich in mesenchymal subtype, the most malignant GBM subtype, while the immunosuppressive cells subpopulation, such as regulatory T cells and myeloid-derived suppressor cells, and anti-inflammatory cytokines, such as interleukin -10, transforming growth factor, beta 1 (TGFB1), are also abundant, altering infiltrative T cells into exhaustion forms. Overall, in GBM, molecular alterations are related with infiltration of T cells, T cells are spare in GBM, compared with another solid tumor, and although in some malignant samples, T cells enrich, the immunosuppressive factors can lead T cells exhaustion. The specific TME and molecular features of GBM provide huge barriers for CAR-T cell therapy.

CAR Structure

Despite various novel CAR designs, four main components exist, which are an antigen-binding domain, a hinge, a transmembrane domain, and an intracellular signaling domain. In every domain, there is a unique function, and an optimal design is needed to perfect CAR efficacy.[37] In tradition, antigen-binding domains of CARs include the variable heavy (V_H) and variable light (V_L) chains of monoclonal antibodies, which are connected by a flexible linker for forming a single-chain variable fragment (scFv).[38] The scFv design, such as the relative position of the complementarity-determining regions, is important to recognize and bind the target antigen.[39] Apart from scFv, other molecules, such as cytokines and ligands, can also be taken as the alternative antigen-binding domains for CARs.[40,41] In addition to working as a bridge to connect extracellular antigen-binding domains to intracellular signaling domains, the hinge and transmembrane domains provide adequate length for facilitating access to the target antigen and enough flexibility to overcome the steric hindrance.[27] The characteristics of the hinge and transmembrane domains could affect antigen binding, signaling transduction, and cytokine production.[42] On the whole, scientists begin to focus on the design of this region to perfect CAR-T cell therapeutic effect.[43–45] Additionally, involving an activation domain and one or more co-stimulatory domains, intracellular signaling domains play a key role in CAR-T cell activation and persistence. The cytokines production and CAR-T cell proliferation are ensured by combining co-stimulatory domains and activation domains.[46] Recently, among GBM, scientists improve CAR-T cells in various aspects innovatively, following the four components of CARs above and the summary of study success and failures on CAR-T cells. This is to improve the efficacy of CAR-T cell therapies for GBM patients while maintaining safety.

The Therapeutic Effects and Limitations of CAR-T in Published Clinical Trials

Up to date, there are published results from clinical trials of CAR-T therapy for interleukin 13 receptor *a*2 (IL13R*a*2), epidermal growth factor variant III (EGFR vIII), human epidermal growth factor receptor 2 (HER2), disialoganglioside

(GD-2), and erythropoietin-producing hepatocellular carcinoma A 2 (EphA2) targets (Table 1). Despite the safety of CAR-T for GBM, there are no successful CAR-T Phase III clinical trials for GBM. In some Phase I CAR-T clinical trials, some patients receive prognostic improvement, but the sample size is small. In addition to the small sample size, these clinical trials show much limitation, such as antigen escape and activation of immunosuppressive molecules. For instance, in the clinical phase I trial (NCT02209376) by O'Rourke et al[47] conducted a first-in-human study of intravenous delivery of a single dose of autologous T cells redirected to the EGFRvIII mutation by a CAR on 10 recurrent GBM (rGBM). The authors demonstrate intravenous delivery CAR-T cells is safe, with median overall survival (OS) of about 8 months in the 10 rGBM. Dependent on 7 samples receiving post-CAR-T surgical resection, CAR-T could traffic to the tumor TME with active GBM, while 5 of the 7 patients undergo EGFRvIII loss after CAR-T infusion. Moreover, after CAR-T infusion, in situ analysis of the TME discovers significant increase in inhibitory molecules, such as PD-L1, indoleamine 2,3-dioxygenase 1 (IDO1) and infiltration of Tregs. Besides, in another phase I trial performed by Stephanie L. Goff et al[48] 18 rGBM patients receive EGFRvIII targeted CAR-T cells after lymphodepleting chemotherapy and intravenous IL2 delivery to support post-transfer. The median progression-free-survival (PFS) is 1.3 months, and the median OS is 6.9 months, the results do not demonstrate clinically meaningful impact in included patients. To sum up, antigen escape, enrichment of immunosuppressive immune cells and cytokines, and the infiltration and persistence of CAR-T affect the efficacy of CAR-T. Next, the limitations of CAR-T cell therapies for GBM are summarized.

Limitations of CAR-T Cell Therapies for GBM

Treatment-Associated Toxicities

Although adverse effects are reported to be relatively uncommon in GBM compared with those in hematological malignancies,[40] the CAR-T cell treatment-related toxicities cannot be lost sight, majorly involving cytokine release syndrome (CRS) and central nervous system (CNS)-specific complications. Through summarizing 8 studies, including 63 recurrent GBM (rGBM) patients receiving CAR-T therapies, a published systemic review and meta-analysis finds that 6 (9.5%) patients suffered from CRS (4 grade ≤2 and 2 grade 4).[53] CRS is characterized by the systemic elevation of numerous cytokines like IL-6 and interferon-γ (IFN-γ).[54] The clinical manifestations of CRS occur rapidly after receiving CAR-T cell therapies, and intensive care unit (ICU) management plays a necessary role in some cases because of respiratory distress and renal dysfunction. What's more, some patients will leave evidence of organ damage, even though the cytokines return to normal.[55] Patients with larger tumor burden and higher doses of infusion of CAR-T cells, which can induce robust immune activation, are more likely to suffer from CRS.[55,56] Taking into account the central role of IL-6 in CRS, anti-IL-6 antibody tocilizumab and dexamethasone are usually used to alleviate inflammation,[47,54] although these drugs may offset the antitumor effects of CAR-T cells.[57] Apart from CRS, it can be found that complications in CNS, called neurotoxicities, such as seizure, intracerebral edema, and language dysfunction.[58,59] In 2022, Majzner et al define "tumor inflammation-associated neurotoxicity" (TIAN) as that unique and specific toxicity profile in patients with primary brain tumors and neurological symptoms associated CAR-T mediated inflammation in CNS tumors. The first classification is related to an increase in intracranial pressure, while the second classification is related to a primary dysfunction of spinal or brain cord structures.[51] In the year of 2017, O' Rourke et al publish a pivotal phase I clinical trial about EGFRvIII targeting CAR-T and reveal that 3 of 10 rGBM experience grade 3 seizure, grade 4 cerebral edema, altered mental status, as well as grade 3 intracerebral hemorrhage.[47] In addition, Ahmed et al report the safety and feasibility of HER2-directed CAR-T therapy in 17 patients (who are 7 pediatric patients and 10 adult patients), and the results indicate that only 3 grade 2 CNS complications events caused by CAR-T infusion, including 2 grade 2 seizures and 1 grade 2 headache.[50] In summary, the treatment-associated toxicities in GBM cannot be ignored, although it is uncommon. The lower rate of adverse events in GBM may be linked with limited efficacy. Although we are committed to improving the efficacy of CAR-T, there is growing importance on the optimal management of CAR-T cell therapy-related toxicities.[55]

Antigen Escape

The proposed challenge in CAR-T cell immunotherapy of GBM is choosing tumor antigens widely expressed on the cell surface, and due to safety reasons, they are not highly expressed in the normal brain or some other life-sustaining

Table 1 Published Clinical Trials of CAR T Therapy in GBM

Target	Study	Study Population	Route of Delivery	Toxicity	Antigen Escape	Outcome	Phase	Reference
IL-13Rα2	Brown et al, 2015[49]	rGBM (n=3)	Intracavitary	Grade 3 Headache Grade 3 Shuffling gait and tongue deviation Grade 3 Leukopenia, headache, and fatigue	n=1	Median PFS: not reported Median OS: 10.9 months	1	NCT00730613
	Brown et al, 2016[40]	rGBM (n=1)	Intracavitary Intraventricular	Grade 2 Headaches, generalized fatigue, myalgia, and olfactory auras	n=1	CR: 7.5 months OS: not reported	1	NCT02208362
EGFRvIII	O'Rourke et al, 2017[47]	rGBM (n=10)	Intravenous	Grade 4 Cerebral edema Grade 3 Seizure and altered mental status Grade 3 Intracerebral hemorrhage	n=5	Median PFS: not reported Median OS: 8.3 months	1	NCT02209376
	Goff et al, 2019[48]	rGBM (n=18)	Intravenous	Grade 3 motor weakness and urinary incontinence Grade 2 neurologic symptoms or seizure (10/18)	NA	Median PFS: 1.3 months Median OS: 6.9 months	1, 2	NCT01454596
HER2	Ahmed et al, 2017[50]	rGBM (n=17)	Intravenous	Grade 2 seizures (2/17) Grade 2 headaches (1/17)	NA	Median PFS: 3.5 months Median OS: 11.1 months	1	NCT01109095
GD-2	Majzner et al, 2022[51]	H3K27M_mutant DMG (n=4)	Intravenous Intraventricular	Increased ICP Hydrocephalus Worsening baseline neurological symptoms Headache	NA	Median PFS: not reported Median OS: 7.8 months	1	NCT04196413
EphA2	Lin et al, 2021[52]	rGBM (n=3)	Intravenous	Pulmonary edema, occurrence of high fever, elevations of the relevant cytokines (2/3)	NA	OS: 86–181 days	1	NCT03423992

tissues.[60] Based on the above criteria, several antigens, such as EGFR vIII, HER2, IL13R*a*2, and EphA2, are the most suitable CAR-T cell targets for GBM.[57] According to expression features of antigens, these preferred antigens could be classified into tumor-restricted antigens expressed merely in tumor cells, and tumor-associated antigens highly expressed in tumor cells and sparsely expressed in normal cells. Unfortunately, under the stress of a single antigen that targets CAR-T cell therapies, GBM tumor cells will evolve to partial/complete loss of target antigen expression, leading to therapeutical resistance, which is known as antigen escape. For example, in 2017, the first-in-human clinical trial (NCT02209376) of CART-EGFRvIII (positive in around 30% of the newly diagnosed GBM samples) outcomes in 10 rGBM patients with EGFRvIII-positive is published. Among them, 5 of 7 patients receiving post-CART-EGFRvIII surgical intervention is with antigen decrease.[47] In the same way, the utilization of CAR-T cells targeting IL13R*a*2 induces the downregulation of IL13R*a*2 expression in GBM. Unfortunately, antigen escape is inevitable in current ongoing CAR-T cell clinical trials, and this mechanism contributes to the primary and secondary resistance for CAR-T cell therapies (Figure 1).

CAR-T Cell Trafficking and GBM Infiltration

First, GBM is tumor in the CNS, and peripheral blood administration is the traditional CAR-T delivery method. The blood circulation to the CNS should pass through the highly selective BBB, which consists mainly of monolayers of endothelial cells, ependymal cells and tanycytic cells connected by restrictive tight junctions.[61] In addition to the physical blockade, a tumor endothelial barrier is commonly present in the tumor vasculature of solid tumors to inhibit immune cell infiltration.[62] In prior research, it is found tumor vasculature can reduce immune cell extravasation and infiltration into tumors by downregulating intracellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1

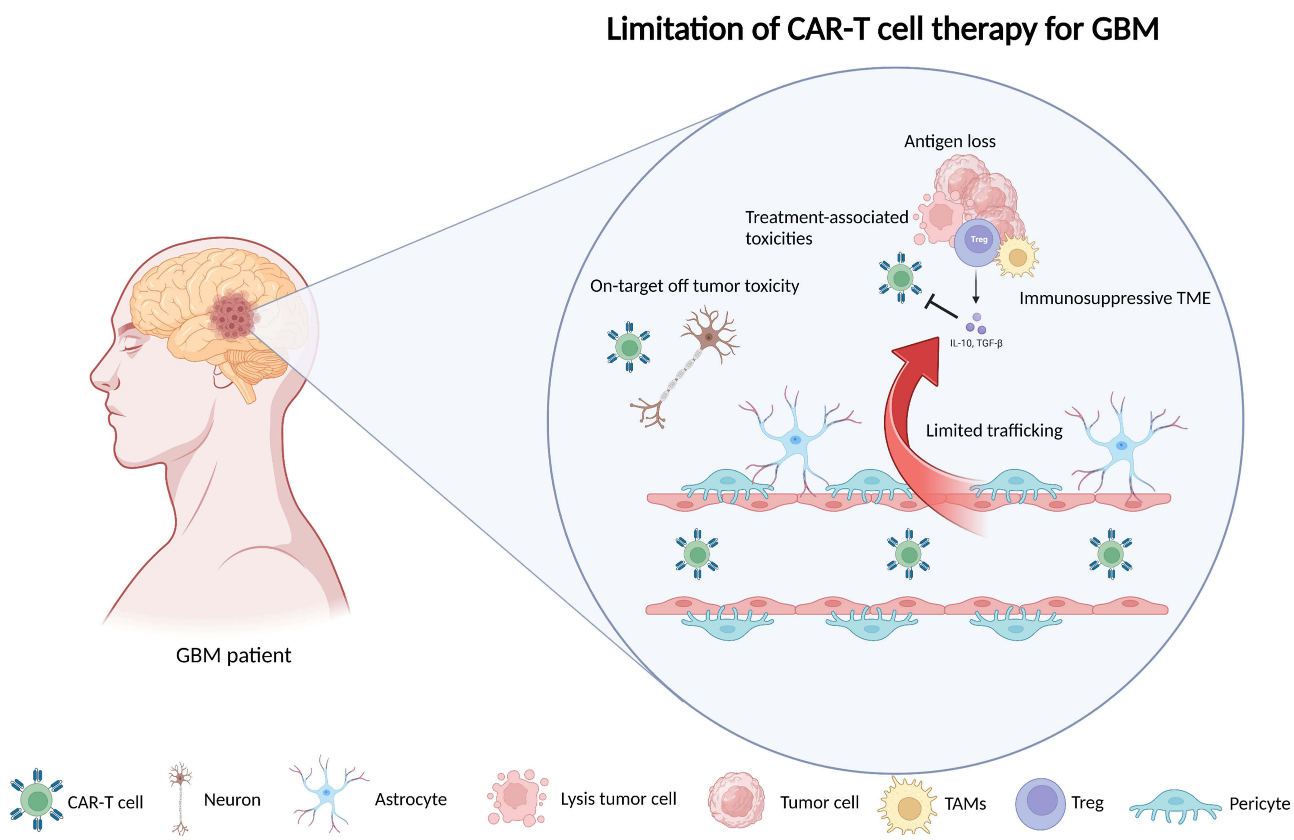

Figure 1 The limitation of CAR-T cell therapy for GBM. 1. The BBB and the unique anatomical boundary limit the CAR-T trafficking into tumor site; 2. The infiltration CAR-T can trigger treatment-associated toxicities, such as CRS, tumor inflammation-associated neurotoxicity (TIAN) and on-target off tumor toxicity; 3. The immunosuppressive TME can educate CAR-T into exhaustion type and inhibit the function of CAR-T cells; 4. Under the treatment pressure of CAR-T, the tumor cells will occur antigen loss.

(VCAM1).[62] Also, characteristics, such as slow and irregular blood flow in tumor vessels, help to create a hypoxic tumor microenvironment, which affects immune cell function.[63] Apart from this, for human and mouse GBM metabolomic and transcriptomic studies, it is found phosphoglycerate dehydrogenase (PHGDH)-mediated endothelial cell (EC) metabolism can promote the formation of a hypoxic and immunodeficient vascular microenvironment driving GBM resistance to CAR-T cell immunotherapy.[64] In addition to the obstruction of the BBB, the mismatch between tumor-expressed chemokines and T-cell surface chemokine receptors contributes to the limited infiltration of T cells in solid tumors.[65] Chemokines which play an important role in T cell recruitment include CXC ligands (CXCL) 9, 10, 11, 16, CCL5, CCL21, and CCL27,[66] and enhanced expression of these chemokines in solid tumors has helped to treat metastatic melanoma, lung cancer, and colorectal cancer.[67–69] In 2019, Jin et al significantly enhance CAR-T cell infiltration and persistence in tumors by both increasing IL-8 expression in glioma and constructing CAR-T cells with IL-8 receptors (CXCR1 or CXCR2) in a preclinical model of glioma.[70] In summary, there is a "cold tumor" in GBM because of the lack of infiltration of T cells,[71] thus leading to CAR-T cells insufficiently encountering tumor cells (Figure 1).

Immunosuppressive Microenvironment

Although regional CAR-T cell therapies are used, the limitation of CAR-T cell trafficking and GBM infiltration still exists (see below). Another tricky barrier is the immunosuppressive tumor microenvironment, which is an obvious difference between hematological tumors and solid tumors. Among GBM, TME is a complicated milieu that includes factors that can regulate tumor proliferation, chemokines, nutrients, and other non-cancerous cell types like fibroblasts and immune cells.[72] GBM-intrinsic and local adaptive mechanisms contribute to immunosuppressive TME.[73] In numerous research, GBM-infiltrating lymphocytes are defined by both an increased prevalence of PD-1 marker expression and a decreased cytotoxic function. This phenomenon indicated that the GBM-specific microenvironment could convert antitumor T cells into exhaustion status.[26,74] Similarly, Shi et al publish that an immunosuppressive GBM environment limits the antitumor ability of CAR-T cells, and it is found in teamwork EGFRvIII-CAR-T cells with PD-1 knockout improved the antitumor activity.[75] Taken together, the immunosuppressive microenvironment may result from the oncogenic, genetic, and epigenetic deregulation of GBM cells, various tumor-supportive immune cells, including TAMs and Tregs, and soluble factor inhibitors, including IL-10 and TGF-β to limit CAR-T function (Figure 1).

Strategies to Enhance CAR-T Cell Function in GBM

Development of Novel CAR Antigens

Novel Target Antigens in GBM

The ideal targets for GBM CAR-T cell therapy are those highly expressed in GBM cells but not within the healthy brain cells. Meanwhile, these targets could significantly induce malignant phenotypes.[76,77] Till now, in the ongoing clinical trials, EGFR vIII, HER2, and IL-13 Rα2 are the most popular target antigens in GBM CAR-T cell therapies, while there are still some limitations to GBM treatment. Recently, advanced CAR-T cells with novel targets, such as B7-H3, CLTX, NKG2DLs, and CD133, have been designed (Figure 2A).

CD133 CAR-T Cells

Cancer stem cells (CSCs) play an important role in chemo- and radio-resistance in various malignant cancers, like GBM, because the self-renewing characteristic of CSCs may induce tumoral heterogeneity.[78] It is reported that the first-line therapies of GBM including TMZ and radiotherapy could drive CSCs accumulation in GBM TME, hence indicating the potential therapeutic effectiveness of CSCs target therapies.[79] CD133 is an important marker of CSCs.[80] In pan-cancers, CD133 is a star molecule that can be applied to design CAR-T cells. In cholangiocarcinoma, it is found that the fourth-generation CAR-T cells targeting CD133 received an effective antitumor response, with higher secretion of IFN-γ and TNF-α.[81] Additionally, promising antitumor activity and a manageable safety profile are proposed by the single-arm, open-label, Phase II trial of CD133-targeting CAR-T cell treatment of hepatocellular carcinoma.[82] Nowadays, multiple CD133-targeting immunotherapies for GBM have received promising clinical outcomes in preclinical models.[78] Among GBM, Zhu et al found that CD133-specific CAR-T cells significantly kill patient-derived GBM stem cells.[83] Besides, Vora et al developed an advanced structure of CAR-T cells targeting CD133 (which is CART133). Through replacing the

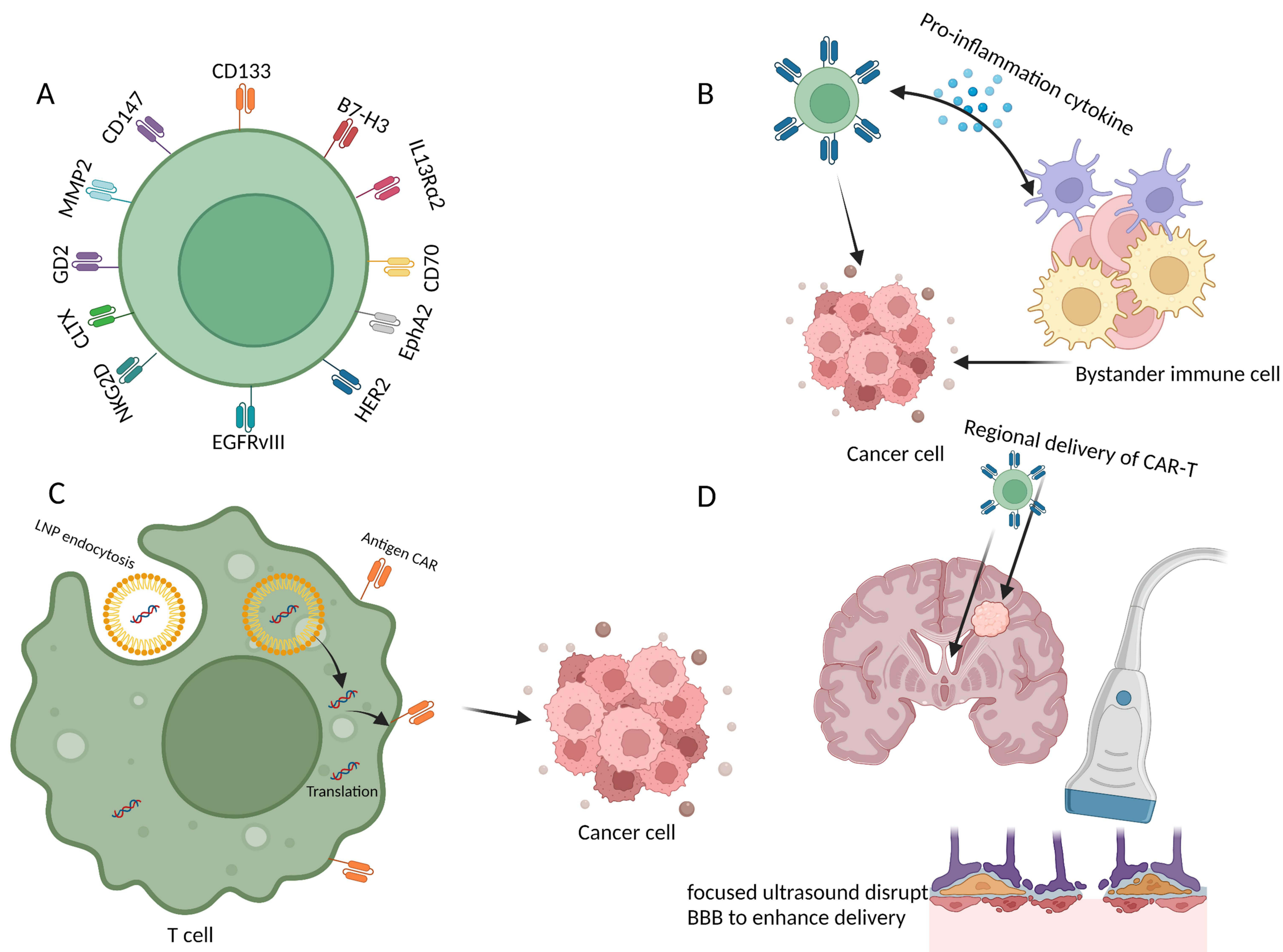

Figure 2 Strategies to enhance CAR-T cell function in GBM. (**A**) Novel target antigens for CAR-T design in GBM; (**B**) Taking advantage of bystander immune cells to enhance antitumor activities; (**C**) Multifunctional mRNA-based CAR-T cells; (**D**) Regional delivery of CAR-T cells and the use of focused ultrasound could influence immune cell activation and CAR-T delivery.

prior scFv domain with the anti-CD133 immunoglobulin G (RW03-IgG) scFv binding to human CD133, the robust and antigen-specific CD133 CAR-T cells respond upon contact with $CD133^+$ tumor cells.[78] Moreover, a pilot clinical research that can be carried out to evaluate the safety and efficacy of CD133 CAR-T cells is in the recruitment phase for patients with recurrent malignant gliomas (NCT03423992) at present.

B7-H3 CAR-T Cells

As an immune checkpoint member belonging to the B7-CD28 families, B7-H3 contributes much to inhibiting the T-cell function.[84] There is a wide expression of B7-H3 in GBM cells.[76] These characteristics make B7-H3 an attractive target to design CAR-T cells.[85] Considering the in vitro and preclinical mice models, B7-H3 positive tumor cells can be significantly eliminated by B7-H3-specific CAR-T cells.[85] In addition, in the diffuse intrinsic pontine glioma, it is pointed out by the primary first-in-human bioactivity and safety study that intracranial delivery may induce local immune activation and tumor repression, and that there is feasibility in the repeated intracranial B7-H3-specific CAR-T cell dosing.[86] Importantly, B7-H3 is also an attractive target to design CAR-NK because of its homogenous expression on GBM. Moreover, the cytolytic activity of B7-H3 CAR-NK cells against $B7\text{-}H3^+$ target cells is reported to show a significant increase while co-transducing B7-H3 CAR-NK cells with TGF-β dominant negative receptor can preserve cytolytic function against GBM in the presence of the exogenous TGF-β.[87] Recently, multiple clinical trials (NCT04077866, NCT04185038, NCT04385173) are performed to assess the B7-H3-specific CAR-T cell therapy for malignant gliomas.

CLTX CAR-T Cells

Chlorotoxin (CLTX), which is a 36-amino acid peptide isolated from deathstalker scorpion *Leiurus quinquestriatus* venom,[88] and CLTX GBM-binding potential is first identified through the conjugation with radioisotope ^{131}I.[89] Recently, based on the potential of binding to a great proportion of GBM cells, CLTX-CAR-T cell has been designed for achieving wider and more efficient GBM targeting. What's more, there is no observable off-target effector activity and antigen escape against the normal cell in the CLTX-CAR-T cells, and meanwhile, tumor regression in orthotopic xenograft GBM tumor models is brought about by the treatment with CLTX-CAR T cells.[90] Since CLTX is not expressed by GBM cells, CLTX possesses targeting properties toward GBM cells. As a result, it is enough for CLTX-CAR-T cells to avoid GBM antigen escape and on-target off-tumor effects.[91] What's more. CLTX-CAR-T cells recognize and kill GBM tumors independent of the other GBM-associated antigens.[90]

Natural Killer Group 2 Member D (NKG2D) CAR-T Cells

Increasing evidence demonstrated that NKG2D ligands (NKG2DLs), which are the ligands of NKG2D, are upregulated in GBM tumor cells and stem cells, while NKG2DLs slight expression is shown by just a few normal tissues.[92] Nowadays, NKG2D-expressing CARs are designed to regress NKG2DLs-expressing GBM cells, utilizing the ability of ligands to bind to receptors. The published results are amazing, and NKG2D-CAR-T cells generated high levels of cytokines, perforin, and granzyme B and effectively lysed GBM cells and cancer stem cells in vitro. In vivo, CAR-T cells showed no obvious treatment-related toxicity in treated mice and markedly eliminated xenograft tumors.[93] In addition, clinical trials to explore the clinical efficiency of NKG2D-based CAR-T cells in relapsed or refractory GBM patients (NCT04717999).

Multi-Antigen-Targeted CAR-T Cells

Up to now, despite ongoing numerous approved CAR-T cell-related clinical trials, there is the existence of lots of obstacles that prevent expanding CAR-T cell immunotherapy in GBM, including tumor heterogeneity and antigen escape. Therefore, novel CAR-T cell modalities are urgently needed for overcoming these problems. Recently, investigators have focused on the exploit of multi-antigen-targeted CAR-T cells. Bispecific CAR (BiCAR), and Tandem CAR (Tan-CAR) are defined as bivalent CAR-T cells co-expressing two CARs targeting various antigens on tumor cells.[94] Through the establishment of the Tan-CAR T cells targeting HER2 and IL-13 Rα2, Hegde et al achieved promising results in preclinical models of GBM, which exhibit advanced Tan-CAR T cells offset antigen escape and enhance the antitumor function. Moreover, the authors presented TanCARs engaged both HER2 and IL13Rα2 at the same time through the induction of HER2-IL13Rα2 heterodimers, therefore giving impetus to superadditive T cell activation in case of the concurrent encountering of the two antigens.[95] In addition, bispecific T cell engagers (BiTEs) refer to the bispecific antibodies redirecting T cells to target antigen-expressing tumors, and BiTE-secreting T cells are a useful therapy against GBM.[96] Nowadays, the research published by Bryan D. Choi combines BiTE and CAR-T cell technologies. A bicistronic construct has been cultivated to induce the expression of a CAR-T cell that is specific for EGFRvIII and CAR-T cells could secret EGFR-targeted BiTEs against EGFR-positive GBM cells, which could make up insufficient tumor-killing function of EGFRvIII-specific CAR-T cell because of antigen escape.[97] Although the appliance of bispecific CAR-T cells could enhance T-cell effector functions and offset antigen escape, the clinical impact of bispecific CAR-T cells is limited by the interpatient heterogeneity in surface antigen expression between patients. To solve this problem, a trivalent T-cell product armed with 3 CAR molecules specific for HER2, IL-13 Rα2, and EphA2 encoded by a single universal (U) tricistronic transgene (UCAR-T cells) is produced for the GBM patients.[98] They observed that almost 100% of GBM patients harbored aberrant expression of the above antigens and that UCAR-T cells targeting these 3 antigens could improve the survival time of autologous GBM patient-derived xenografts (PDXs).[98] Besides, the technology of logic gates CAR-T cells, recognizing both priming and killing CAR antigen, is considered another excellent model of multiple antigens CAR-T cells that can achieve equilibrium between antigens specificity and heterogeneity.[99] Nowadays, investigators perfect the specificity and persistent antitumor activity of therapeutic T cells by using the synthetic Notch (synNotch) CAR circuits, which is the synNotch receptor recognizing a specific priming antigen, and next is primed to induce expression of a CAR-directed against killing antigens, such as IL-13Rα2 or CD133, therefore avoiding off-tumor activity and causing improvement of specificity and persistence of T cells directed against GBM.[99,100]

Taking Advantage of Bystander Immune Cells

Recently, the inability of CAR-T cells to sustain effector function and the activation of bystander immune cells in suppressive GBM TME is the main obstacle to CAR-T cell therapy success within GBM patients. Besides, cytokines, produced by various cell populations, are important immunoregulatory elements of TME contributing to the exhaustion of CAR-T cells.[101] In recent years, research in CAR-T cell development, utilizing cytokines to enhance the function of CAR-T cells and bystander immune cells, is underway. Also known as the fourth-generation CAR-T cells, cytokine-secreting CAR-T cells are also a successful strategy.[102] Technically, CAR-T cells are also engineered via the nuclear factor of activated T cell (NFAT)-responsive expression cassette for the inducible expression of the transgenic cytokine, like IL-12, which is a pro-inflammatory cytokine.[102] Additionally, IL-12 could activate T cells, and recruit bystander immune cells against cancer cells without CAR-T cells targeting antigens. Among GBM, Burkhard Becher et al promoted the combination of CAR-T cells and local delivery of IL-12. In the meantime, IL-12 could support the persistent cytotoxic activity of CAR-T cells and reprogram endogenous T cells and myeloid cells against tumor cells, with only mild systemic effects.[103] Besides, to overcome tumor antigen heterogeneity, investigator-engineered T cells secrete dendritic cell (DC) growth factor Fms-like tyrosine kinase 3 ligands (Flt3L) to stimulate the endogenous DCs, and engineered T cells overcome the clinical problem of antigen-negative tumor escape after the adoptive cell therapy[104] (Figure 2B).

Multifunctional mRNA-Based CAR-T Cells

Up to now, major methods of genetically modified CAR-T cells to regress GBM have been through the retroviral vectors or the nonviral transposon-transposase systems to stably integrate transgenes that encode CAR.[105] Despite reaching expression during the long term, they still possess some limitations and safety concerns, including genomic alterations risk bringing about the malignant transformation of T-cell clones.[106] While mRNA-based CAR-T cells possess multiple advantages. Firstly, because of the production efficiency, there can be more easy testing of the iterative changes in the CAR binding site or structure.[107] Secondly, mRNA is easier for clinical transformation and is less expensive compared to viral vectors. Last but not the least, the mRNA transient expression may guarantee safety, especially within the brain where significant morbidity and mortality may be caused by the on-tumor and on-target/off-tumor toxicity.[108] In addition, the transient nature may be a barrier for mRNA-based CAR-T cells to provide persistent tumor elimination,[107] and the repetitive infusion of CAR-T cells may solve this problem. Recently, mRNA-based treatments gradually become attractive means to counter various intractable diseases, such as SARS-CoV-2[109,110] and malignant tumor.[111,112] In numerous research, a therapeutic approach that can produce transient antifibrotic chimeric antigen receptor (CAR) T cells in vivo is developed through the delivery of the modified messenger RNA (mRNA) in T cell–targeted lipid nanoparticles (LNPs), which is called mRNA-based CAR-T cells.[113,114] While mRNA-based CAR-T cells are characterized by rapid, safe, transient, and cost-effective T-cell modification. Meister et al created the multifunctional mRNA-based CAR-T cells which coexpressed NKG2D, IL12, and IFNα2. Importantly, the mRNA-based CAR-T cells decreased T-cell exhaustion, exhibited promising antitumor function, and induced a proinflammatory tumor microenvironment.[115] In addition, mRNA GPC2-redirected CAR-T cells could elevate survival in pediatric brain tumor xenograft models and enhance tumor regression, with no clinical toxicity evidence.[116] To sum up, dependent on mRNA transfection technologies, investigators could create CAR-T cells in vivo with efficient antitumor function and may be the potential to alter the GBM treatment landscape (Figure 2C).

Regional Delivery of CAR-T Cells

Since GBM is a CNS tumor, the intravenous administration approach may have poor utility due to the presence of BBB, which is one of the reasons for the inefficiency of CAR-T therapy for GBM. Recent research about CLTX CAR-T cells treating GBM indicated tumor regression is enhanced by the regional delivery of CAR-T cells, while mice treated intravenously did not receive comparable tumor elimination.[90] Besides, current CAR-T cell-related clinical trials gradually play an important role in regional delivery (intraventricular and/or intracavitary delivery).[94] Meanwhile, a comprehensive review summarizes there is both feasible and safe regional delivery of CAR-T cells in patients with solid tumors, which could generate potent and long-lasting antitumor immunity.[117] Similarly, in the preclinical studies targeting CNS tumors, various regional delivery approaches, including the ommaya device and catheter/reservoir system, show transient antitumor responses, with well-tolerated adverse events.[118] Furthermore, the regional delivery devices enable the repetitive delivery of CAR-T cells to the lateral ventricle and/or tumor cavity and induce the spread of CAR-T cells to CSF circulation, therefore causing systemic immunity. Meanwhile, Brown et al have demonstrated that the peripheral blood in a patient with GBM treated with intraventricular regional therapy showed an absence of cytokines, escaping the cytokine-mediated organ damage. Additionally, using low-intensity pulsed ultrasound (LIPU), which is considered a safe therapeutic method, could temporarily open BBB, and then enhance CAR-T cells delivery to the tumor and surrounding brain parenchyma to treat GBM.[18] In the EGFRvIII-U87 gliomas NSG mice model, CAR-T cell delivery with LIPU-induced BBB disruption led to a remarked increase in CAR-T cell delivery to the CNS and an increase in median survival by greater than 129%, in comparison with the CAR-T cells alone.[18] Apart from the immune cell delivery, focused ultrasound could also influence immune cell activation and tumor antigens release[119] (Figure 2D).

The Combination of CAR-T Cell Therapy with Other Therapies

To enhance the therapeutic effect of CAR-T cells and overcome the limitations of CAR-T cell therapy in GBM, modification of CAR-T cells is one idea, and another idea is to combine existing therapies with CAR-T cell therapy to achieve a synergistic treatment. Currently, there are certain combination therapies with CAR-T cells, which have achieved superior results in comparison with a single therapy, and there are also some trials that have demonstrated the promise of combination therapy in GBM (Table 2).

Combination with Chemotherapy

Although temozolomide (TMZ) is seen to be a commonly used drug in treating glioblastoma, a combination of TMZ and immunotherapy is not promising. This is because TMZ treatment may induce hypermutation in glioblastoma, and hypermutation can adversely affect immunotherapy for GBM by creating resistance and increasing antigen escape.[57,120] However, the inhibitory effect of temozolomide on immunotherapy may be neutralized by the positive therapeutic effect of temozolomide itself, and in preclinical experiments, temozolomide-induced lymphodepletion increased pro-inflammatory factor expression and antigen-specific T cell proliferation in mice, improving the effectiveness of adoptive treatment of brain tumors.[121] Therefore, clinical trials comparing the effects of temozolomide alone and a combination of CAR-T cell therapy with temozolomide still exist (NCT04077866). Despite there being no published

Table 2 Summary of CAR-T Cell Combination Therapy Clinical Trials with Other Cancer Treatments in GBM

Treatment	Combination	CAR T Cell Target Ag	Phase	References
PD-1 inhibitor	Pembrolizumab	EGFRvIII	I	NCT03726515
PD-1 inhibitor and CTLA4 inhibitor	Nivolumab, ipilimumab	IL13Ra2	I	NCT04003649
Lymphodepleting chemotherapy and cytokine	Cyclophosphamide, fludarabine, Aldesleukin (IL-2)	EGFRvIII	I, II	NCT01454596
Cytokine	Aldesleukin (IL-2)	IL13Rα2	I	NCT01082926
Lymphodepleting chemotherapy	Cyclophosphamide, fludarabine	EGFRvIII	I	NCT02844062
		EphA2	I	NCT03423992
		GD2	I	NCT04099797
Standard chemotherapeutic agents of GBM	Temozolomide	B7-H3	I, II	NCT04077866, NCT04385173

results yet, there are positive clinical implications. In patients with malignant lymphocytic leukemia, extensive use of lymphodepleting chemotherapy regimens (cyclophosphamide alone, fludarabine, and cyclophosphamide) before CAR-T cell therapy suppresses the patient's autologous Tregs cell concentration, increases pro-inflammatory factors, and promotes proliferation and stabilization of CAR-T cells.[122–124] As for lung tumors and neuroblastoma, good clinical efficacy has also been shown by lymphodepleting chemotherapy before CAR-T cell therapy.[125,126] During treating glioblastoma, there are currently two clinical studies with published results via the combined therapy of CAR-T cells and lymphodepleting chemotherapy. In the clinical phase I trial of Goff et al, an effective antitumor effect has not been shown by the lymphodepleting chemotherapy and intravenous IL-2 adjuvant anti-EGFRvIII-CAR-T cells therapy.[48] Through lymphodepletion chemotherapy combined with EphA2-redirected CAR-T cells, Lin et al explored the expansion of CAR-T cells in peripheral blood and cerebrospinal fluid for over 4 weeks.[126] Although the experiment is still ongoing, this observation suggests a good clinical effect (Figure 3A).

Combination with Radiotherapy

By mutually reinforcing each other's approach, radiation therapy, and CAR-T cell therapy may act synergistically in combination therapy. In mouse models, CART133 cells showed good efficacy in terms of targeting $CD133^+$ CSCs in patient-derived GBM xenograft models, which are the chemo- and radiation resistance markers of glioblastoma.[78] This implies that applying CART133 cells could optimize the clinical efficacy of standard GBM treatments. In addition, since CAR-T cells can mediate apoptosis of target cells through the Fas/FasL or TRAIL axis engagement,[127] and DeSelm et al

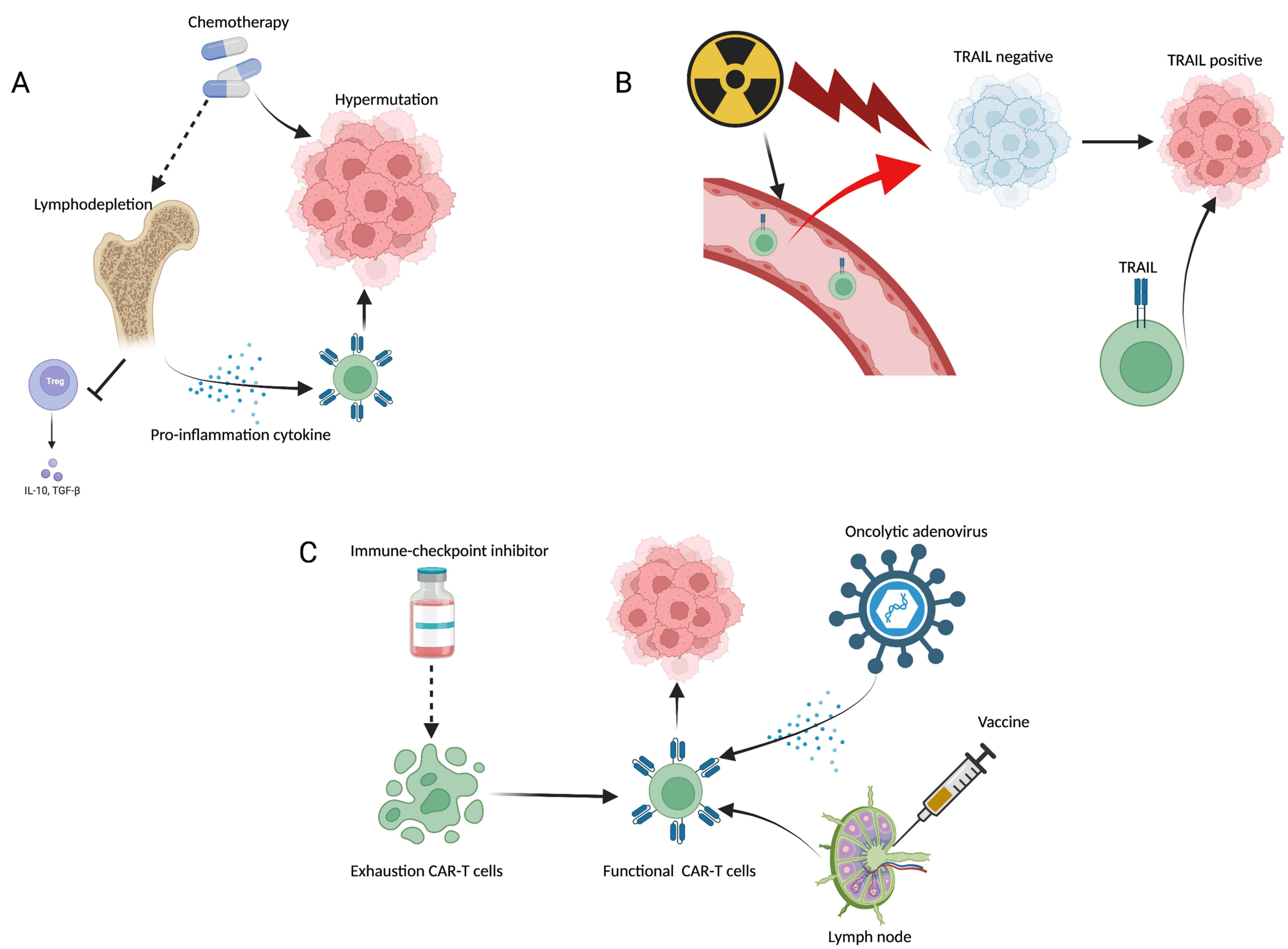

Figure 3 The combination of CAR-T cell therapy with other therapies; (**A**) Chemotherapy could cause lymphodepletion and increased pro-inflammatory factor expression as well as antigen-specific T cell proliferation; (**B**) Radiotherapy could induce the infiltration of CAR-T cells and sensitize antigen-negative tumor cells to CAR-T cell therapy; (**C**) The combination of other immunotherapy could enhance the function of CAR-T cell therapy.

found that low-dose radiotherapy can sensitize antigen-negative tumor cells to TRAIL-mediated apoptosis, CAR-T cells activated by antigen-positive tumor cells expressing TRAIL can resist tumor heterogeneity through killing antigen-negative tumor cells (Figure 3B).[128] In the treatment of brain tumors, it is found radiotherapy increases vascular permeability at the tumor site.[129] In a preclinical study by Murty et al, it is found that radiotherapy allows rapid extravasation of CAR-T cells from the vascular system and expansion in the tumor microenvironment, which can cause a more durable and robust immune response.[130] This feature of radiotherapy may imply its good adjuvant effect on CAR-T cell delivery. Weiss et al found that in preclinical models of glioma, by promoting CAR-T cell migration to the tumor site and enhancing effector function, subtherapeutic doses of local radiotherapy combined with NKG2D-CAR-T cells' treatment generated synergistic activity in mouse glioma models.[131] In addition, Sampson et al found that autologous lymphodepletion with total body irradiation before CAR-T cell treatment upregulated CAR-T cells' pro-inflammatory cytokine levels and increased the CAR-T cell proliferation and survival.[132,133] Overall, there is a good synergy between radiation therapy and CAR-T cell therapy, which can also be found in the former clinical models. In application, there should be more precise limits for the dose and site of radiation therapy. Generally, it is believed that low doses of 2 Gy of radiation favor the formation of an immunogenic tumor microenvironment,[133] while high doses of 5–10 Gy may increase the hypoxia area and limit infiltration of T cells to the tumor, harming immunotherapy.[134] Meanwhile, hypofractionated radiation therapy also favors the maintenance of the suppressive immune microenvironment.[133,135] Thus, more exploration of radiation therapy dose and modality in combining radiotherapy and CAR-T cell therapy is worth to be carried out.

Combination with Other Immunotherapies

As previously mentioned, fourth-generation CAR-T cells secreting cytokines prolong the maintenance of CAR-T cells' effector effects and overcome limitations of the suppressive immune microenvironment.[16] Apart from modifying CAR-T cells themselves, there is a similar adjuvant effect in combination therapy with local delivery of cytokines. IL-15 is an immunostimulatory factor that targets tumor cells. Lanitis et al established a CAR-T cell model co-expressing IL-15 and found that IL-15 can promote CAR-T cells infiltration in tumors, enable enhanced CAR-T cell effector function, and promote NK cell activation and reduction of M2 macrophages in the tumor microenvironment.[136] Besides, IL-15 can prevent T-cell exhaustion and reduced PD-1 expression on the cell surface.[137,138] In addition, Alizadeh et al found the co-culture of IL-15 and CAR-T cells targeting human glioma cell lines could maintain a less-differentiated stem cell memory phenotype through mediating reduced mTORC1 activity, hence showing less differentiation, less depletion, and increased anti-apoptotic properties, and enhanced proliferation after antigen stimulation.[139] The investigators optimized the dosing regimen in a preclinical trial of combined cytokine and CAR-T cell therapy. Tang et al found that protein nanogels (NGs) loaded with IL-15 superagonist (IL-15Sa) equipped with CAR-T cells proliferated with higher tumor specificity and better therapeutic efficacy compared to systemic administration of cytokines, improving survival of glioblastoma mouse model.[140] Through a single intratumoral injection (300 ng) of IL-12 combining EGFRvIII-targeted CAR-T cells to treat a mouse glioma model, Agliardi et al found that IL-12 remodels TME with little systemic cytokine toxicity and enhances CAR-T cells cytotoxicity.[103] Currently, there are two clinical trials in GBM documenting a combination of IL-2(Aldesleukin) and CAR-T cell therapy, and the intratumoral (NCT01082926) and intravenous drug delivery (NCT01454596) methods are used for each, in which the former is reported to be well tolerated for Intracranial administration of CAR-T cells (GRm13Z40-2) with aldesleukin.[141] Due to the suppressive immune microenvironment of GBM, it is theoretically thought that the synergistic use of immune checkpoint inhibitors plays a synergistic role in the CAR-T cell therapy breakthrough in GBM.[142] Traditionally, PD-1 is a marker of T-cell exhaustion, and PD-1 inhibition serves as a therapeutic tool to increase T-cell toxicity and keep the T-cell activation state.[143] In GBM models, it is thought that PD-L1/PD-1 axis blockade can improve T-cell function by reducing Treg expansion.[144] In the treatment of metastatic melanoma, PD-1-targeted combination therapy could have a synergistic effect on the antitumor effects of CAR constructs.[145] On this basis, although published results are not yet available, there are clinical trials of CAR-T cell and immune checkpoint inhibitors combination therapies to treat GBM (NCT03726515, NCT04003649). However, there are positive voices for anti-PD-1/PD-L1 in GBM. Gargett et al analyzed samples from a recurrent GBM clinical trial (NCT02209376). The results showed that PD-1 expression in CAR-T cells may predict a better prognosis for GBM patients after receiving CAR-T cell therapy through a positive regulation of AUC (concentration in peripheral blood) of CAR-T cells in peripheral blood and PFS of patients.[143] This result may be related to the complex mechanism of the PD1-PDL1 pathway in gliomas.[143] In previous experiments, it has been confirmed that PD-1$^+$ T cells have higher expression of activation and depletion markers within gliomas and that compared to its PD1 counterpart, there is higher T cell receptor diversity and IFN-γ production in this population.[143,146] Therefore, further clinical controlled trials are still needed to validate the combination of immune checkpoint inhibitors and CAR-T cell therapy. Apart from this, combination therapy of immunomodulatory agents and CAR-T cells has been studied. Lenalidomide (Thalidomide analog) inhibits the inhibitory effects of CTLA4-Ig on T-cell proliferation and cytokine production, increases the number and activity of T cells and natural killer cells, and directly enhances antibody-dependent cell-mediated cytotoxicity (ADCC) by using the increase of the secretion of IL-2 and INF-γ.[137,138] In 2015, Kuramitsu et al demonstrated that lenalidomide induced the proliferation of EGFRvIII-specific CAR-T cells target in a mouse glioma model and enhanced immune synapses between effector and target cells.[147] In 2020, it was found by Wang et al that lenalidomide enhanced CD133-specific CAR-T cells cytotoxicity in glioma cell lines, possibly regulating CAR-T cell function by inducing degradation of transcription factors *Ikaros* and *Aiolos*.[148] Therefore, the use of modest immunomodulatory elements could be a new direction in subsequent clinical trials of CAR-T cell combination therapy (Figure 3C).

Combination with Vaccines

The idea of cancer vaccine therapy, especially therapeutic vaccines, mainly causes T-cell immune response.[149] When used with CAR-T cell therapy, its main role is promoting the proliferation and killing efficacy of CAR-T cells. Regarding treating GBM, it can be found that the related studies are still at the stage of preclinical trials. In the case of peptide vaccines, Ma et al designed an amphipathic CAR-T cell ligand (amph-ligand) as a vaccine, which is transported to lymph nodes after injection and modified antigen-presenting cell surface. Thus, this can initiate CAR-T cells in the natural lymph node microenvironment and trigger massive CAR-T cells expansion, which can demonstrate stronger antitumor efficacy compared to the single CAR-T cell treatment in an EGFRvIII mouse glioma model[150] (Figure 3C). Additionally, in cellular vaccines, using radiation-induced immunogenic cell death of glioma stem cells as vaccines, Sun et al confirmed that enhanced recognition of tumor stem cells by CAR-T cells promotes proliferation and antitumor effects of CAR-T cells.[151] Apart from this, in the immunotherapy approach for glioblastoma proposed by Altinoz et al, the authors proposed to make the GBM patients being injected with lysates of their tumor cells and lysates of the GBM cell line U251 before the CAR-T cell treatment. This allows transgenic T cells to show greater potency with and through intercellular interactions with native immunocytes.[152] The therapeutic vaccines are mainly injected in peripheral blood, whereas as mentioned earlier, CAR-T cell therapy is administered locally in glioblastoma. In combining tumor vaccines and CAR-T cell therapy, attention should also be paid to the exploration of the dosing modality.

Combination with Oncolytic Adenovirus

Oncolytic adenoviruses (oAD) refer to viruses that can specifically infect tumor cells, directly lyse them, and induce endogenous antitumor immune responses. Because of their specificity, they can also be used as a platform for targeted tumor delivery of drugs.[153] They can be genetically engineered to express pro-inflammatory cytokines,[154] small molecule immune checkpoint inhibitory antibodies,[155] BiTEs,[156] and some other structures to act in concert with CAR-T cell therapy (Figure 3C). Through the establishment of an interleukin-7-loaded oncolytic adenovirus (oAD-IL7) and B7H3-targeted CAR-T cell, Huang et al pointed out that for in vitro and in vivo experiments in glioblastoma, oAD-IL7 inhibits CAR-T cell apoptosis and enhances CAR-T cell proliferation, with better-combined efficacy.[157] Recently, Evgin et al demonstrated the therapeutic efficacy of CAR-T cells is significantly optimized in a glioma mouse model by the advance loading of CAR-T cells with reovirus and restimulating them with reovirus 8 days after treatment.[158] There should also be clinical trials on combining oncolytic adenovirus and CAR-T cells, and further exploration of the mode of administration in mouse models is worthwhile.

The Overview of Promising CAR-T Cells Therapies Strategies

Although most of the CAR-T studies in glioma are currently at the stage of preclinical studies or starting clinical studies but not publishing results, there are still some promising trials in CAR-T cell therapy where results have been reported. For systemic administration, in the phase I clinical trial of Ahmed et al[50] on CAR-T cells targeting HER2, authors utilize autologous modified virus-specific T (VST) cells specific for cytomegalovirus, Epstein–Barr virus, or adenovirus to enhance the CAR-T persistence. This clinical trial involves 17 patients with progressive GBM. Patients receive 1 or more infusion of autologous HER2-CAR VST ($1\times10^6/m^2$ to $1\times10^8/m^2$) without prior lymphodepletion, and CAR-T cells can be detected for 12 months in the peripheral blood, which is longer than another research.[48,159] The median OS is 11.1 months from the first T-cell infusion and 24.5 months from diagnosis, indicating the clinical beneficial. Moreover, Ahmed's team successfully constructed bispecific CAR-T cells targeting IL13Rα2 and HER2,[95] and this multi-targeted CAR-T cells that can compensate for the defect of antigen escape may be a hope for future applications regarding CAR-T in glioma. The research in preclinical GBM mice models demonstrates that intravenous delivery of CAR-T cells does not significantly prolong survival, while intraventricular infusion with CAR-T is efficacious against GBM,[19] indicating the value of regional delivery. In the phase I clinical trial of Brown et al on CAR-T cells targeting IL13Rα2, 3 rGBM patients receive 12 regional deliveries at a maximum dose of 10^8 CAR-T cells targeting IL13Rα2 via a catheter/reservoir system. Although the sample size is too small, 3 patients with relapsed GBM attain a median overall survival time of 10.9 months (NCT00730613).[49] For preclinical studies, recently, addition of Nivolumab, an anti-PD-1 monoclonal antibody, can promote cytotoxicity of GD2 CAR-T in vitro, and in orthotopic NOD/SCID GBM animal model, combination of GD2 CAR-T and Nivolumab can expand the survival time.[160]

Summary and Discussion

Recently, although there is increasing attention on the appliance of CAR-T cell therapies for GBM, the clinical outcome is still dismal. The obstacle of BBB impedes the infiltration of CAR-T into GBM TME. Besides, extensive heterogeneity of tumor subpopulation can enhance the tumor recurrence under the stress of CAR-T therapies. For the characteristics of tumor TME, after CAR-T infusion, the permeation of suppressive immune cell subpopulations, such as Tregs and TAMs and the secretion of immunosuppressive cytokines, can limit efficacy of CAR-T therapy. These challenges restrain the appliance of CAR-T therapy among GBM patients. Up to date, for GBM, there is a lack of two arms large-scale clinical trials to compare the efficacy of CAR-T therapy. According to the data from complete clinical trials, the systemic and regional delivery of CAR-T cells is safe and feasible, with controlled complication, while antitumor function of single CAR-T therapy is limited. Frontier research is working on designing and combining novel antigens, applying regional CAR-T cell delivery, activating bystander immune cells and TME, developing multifunctional CAR-T cells via mRNA transfection technology, and combing promising methods to overcome the challenge in GBM. Encouragingly, there is preliminary efficiency in preclinical practice in the advanced strategies, and need further demonstration in clinical trials. Although multiple immunotherapies are demonstrated to be safe for GBM samples, the feasible for treatments combination remain unclear. The ongoing clinical trial (NCT04003649) aims to recruit 60 GBM patients to elevate the efficacy and safety of combination of CAR-T cells targeting IL13Rα2 with nivolumab and ipilimumab. Moreover, the individualized treatment may be the future trends in GBM CAR-T therapy. Haruhiko Kishima et al establish a library of monoclonal antibodies against tumor cell lines derived from GBM samples, and they find E61 and A13, two antibodies, which can bind with tumor cell lines from most GBM patients, but not with nonmalignant human brain cells. In the future, more antibodies against multiple GBM samples and novel target antigens will be found via this method.[161] Accordingly, combination and multidisciplinary immunogenic regimens, and more precious technology may provide new avenue for CAR-T-related treatments and need to be verified in two-arm large-scale clinical trials.

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